

EXPOSURE OF MIGRATORY SHOREBIRDS TO ORGANOPHOSPHORUS AND  
CARBAMATE PESTICIDES AT MIGRATORY STOPOVER AND NON-BREEDING SITES  
IN THE WESTERN HEMISPHERE

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## Abstract

Monitoring programs indicate that numerous shorebird populations are subject to ongoing declines. The U.S. Shorebird Conservation Plan lists twenty-seven shorebird species as species of high concern and seven as highly imperiled, including the Buff-breasted Sandpiper (*Tryngites subruficollis*). One hypothesis for ongoing population declines is exposure to toxic chemicals and pollutants. The purpose of this project was to characterize plasma cholinesterases (ChEs) of migratory shorebirds and address potential exposure to organophosphorus (OP) and carbamate (CB) pesticides. Consumption or contact with these pesticides can cause mortality and a variety of sub-lethal effects. Buff-breasted Sandpipers and other upland shorebirds are particularly likely to encounter agrochemicals due to their habitat use at the non-breeding grounds. I sampled migratory shorebirds over three seasons, during north- and southbound migration in 2006 and 2007 in Texas, Kansas, and Nebraska and during the non-breeding season in 2007 in Argentina, Uruguay, and Paraguay. I collected blood samples and footwashings from reference sites, where OP and CB pesticides were not used, and agricultural sites, where these two insecticides were recommended for control of crop pests. I assessed several variables known to affect plasma ChE activity including body size, date of capture, time of capture, condition, sex, and region. Small-bodied species had higher levels of ChE activity in plasma than large-bodied species. Plasma ChE activities varied with date of capture in 3 of 5 species sampled in North America. Sex differences were significant in 1 of 4 species tested. Plasma acetylcholinesterase (AChE) activity was higher among White-rumped Sandpipers sampled in North America but there was no difference between regions among Buff-breasted Sandpipers.

Time of capture and individual condition did not affect plasma ChE activity. Estimates of exposure to ChE inhibitors were addressed in five species. Plasma AChE and butyrylcholinesterase (BChE) activities of Buff-breasted Sandpipers were lower at agricultural sites in South America but BChE activity was higher at agricultural sites in North America. There were no differences between sites in four other species tested. A meta-analysis across all species indicated that in 4 of 6 comparisons habitat type had a negative effect on AChE activity consistent with exposure to ChE inhibitors but there was a regional positive effect of agricultural habitat on BChE activity in North America. Comparison of body mass between sites suggested that use of habitats with potential pesticide application did not affect mass gain. Project results suggest that 1 of 5 shorebird species tested was exposed to ChE-inhibiting pesticides at the non-breeding grounds and future monitoring is necessary to assess potential effects at the population level. This study highlights the importance of complete sampling and addressing variability in plasma ChEs before making estimates of exposure to OP and CB pesticides. It provides the first estimates of migratory shorebird exposure to OP and CB pesticides, a potential conservation issue. Future research should include continued monitoring of Buff-breasted Sandpiper ChE levels and habitat use. Other sources of anthropogenic declines such as habitat loss and illegal hunting should be investigated for species that did not show evidence of exposure.

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## **CHAPTER 1 - Introduction**

Nearctic-Neotropical migratory shorebirds represent a taxonomic group with diverse migration strategies and natural histories (Page and Gill 1994). In the Western Hemisphere, long-distance migrants breed on the arctic tundra and spend the austral summer in Central and South America while some short-distance migrants do not venture outside of North America. Migration along the coast is characterized by large aggregations of shorebirds using a few important stop-over sites where they exploit abundant, seasonal resources. Shorebird migration in the interior of North America follows a broad-front pattern and shorebirds are more dispersed on the landscape to exploit variable resource availability (Skagen and Knopf 1993).

The conspicuous nature of shorebird migration and reliability of arrival time caused many species to suffer population declines after exploitation for food and sport in the early 19<sup>th</sup> century (Forbush 1916, McIlhenny 1943). Although hunting of most species of shorebirds is now illegal, many species continue to experience population declines. According to the U.S. Shorebird Conservation Plan (Brown et al. 2001), approximately 45% of migratory shorebird species are declining and recent analyses of survey data continue to indicate similar trends (Morrison et al. 2006, Bart et al. 2007).

Several factors have been implicated in on-going shorebird population declines. Anthropogenic factors include exposure to chemicals and pollutants, habitat degradation and destruction, and continued illegal hunting (Senner and Howe 1984, Zöckler et al. 2003). The goal of my thesis was to evaluate migratory shorebird exposure to one group of environmental contaminants, organophosphorus and carbamate pesticides using plasma cholinesterase (ChE) activity. Specifically, I aimed to 1) assess variation in plasma ChEs of migratory shorebirds

during migration and at non-breeding sites, 2) provide reference values of plasma ChEs from North and South America for this and future ecotoxicological studies, and 3) evaluate exposure of migratory shorebirds to organophosphorus and carbamate pesticides during migration and at the non-breeding grounds. The results are applicable to current topics in shorebird conservation.

This thesis is organized into four core chapters. Here, I introduce the study. In chapter two, I evaluate intrinsic and extrinsic variability of plasma ChEs of migratory shorebirds captured during migration in North America. I provide reference values of plasma ChEs for migratory shorebirds sampled during northbound migration in North America. In chapter three, I provide reference values of plasma ChE activity for migratory shorebirds at non-breeding sites in South America. I evaluate shorebird exposure to ChE-inhibiting pesticides by comparing ChE activity in samples collected from agricultural habitats versus samples collected from reference sites throughout the Western Hemisphere. The last chapter, chapter four, is an overview of findings and major conclusions of my study.

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## **CHAPTER 2 - Plasma cholinesterases for monitoring pesticide exposure in Nearctic-Neotropical migratory shorebirds<sup>\*</sup>**

Khara M. Strum, Matilde Alfaro, Ben Haase, Michael J. Hooper, Kevin A. Johnson, Richard B. Lanctot, Arne J. Lesterhuis, Leticia López, Angela C. Matz, Cristina Morales, Benjamin Paulson, Brett K. Sandercock, Julian Torres-Dowdall, and María Elena Zaccagnini

### **Abstract**

Organophosphorus (OP) and carbamate (CB) pesticides are commonly used agrochemicals throughout the Western Hemisphere. These pesticides have caused mortalities in migratory birds and adverse physiological effects in trials with captive birds. Migratory shorebirds use a variety of habitats during the austral summer in temperate South America and during migration through the Great Plains of the United States. Habitats where risk of exposure is high include rice fields and turf grass farms where agrochemicals are used. Cholinesterase (ChE) is a specific biomarker for monitoring OP and CB exposure and can be measured using standard laboratory procedures. Plasma ChE activity is useful as a non-lethal means of monitoring avian exposure to OP and CB pesticides. Many variables can affect enzyme activity and reactivation assays are not always possible, thus reference values of ChE activity are a necessary component of monitoring exposure. During northbound migration in 2006, we sampled four upland and five wetland shorebird species at four pesticide-free sites in North America, characterizing and measuring plasma ChEs in all shorebird species. Small-bodied species had higher levels of ChE activity in plasma than large-bodied species.

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Acetylcholinesterase (AChE), the enzyme whose inhibition leads to symptoms of poisoning, showed less inter-specific variation than butyrylcholinesterase (BChE). Plasma ChE activities varied with date of capture in 3 of 5 species. Sex differences in BChE were significant in 1 of 2 species tested. Our baseline ChE values for migratory shorebirds provide a framework for future ecotoxicological studies of Nearctic-Neotropical migrant shorebirds.

## **Introduction**

Organophosphates (OPs) and carbamates (CBs) averaged 68% of the active ingredients in insecticides used in the United States from 1980 through 2001 (Kiely et al. 2004). The use of OPs and CBs increased in the 1970's after organochlorine pesticides (e.g., DDT) were banned due to health and environmental hazards (e.g., Henny and Bennett 1990). OPs and CBs provide an alternative to the environmental persistence and bioaccumulation of organochlorines (Blus 2003). In spite of their limited persistence in the environment, many of these chemicals are highly toxic to avian species and incidental kills of migratory birds are well documented (Basili and Temple 1995, Goldstein et al. 1999a). Mass mortality incidents have resulted in public awareness campaigns that emphasized the toxicity of OP and CB pesticides, and in some countries, lead to laws against the use and manufacture of some of these pesticides (Hooper et al. 1999, Hooper et al. 2003).

Although many highly toxic OPs and CBs are prohibited or highly regulated in the Americas (Anonymous 2004, USEPA 2007), instances of mortalities and high level exposures have been reported recently (Pain et al. 2004, Wobeser et al. 2004, Renfrew et al. 2006). Furthermore, less toxic OPs and CBs continue to be used in agriculture throughout North and South America. For example, chemicals that inhibit cholinesterase (ChE) are part of the rice cultivation industry in Uruguay and Argentina (Garamma et al. in Blanco et al. 2006a, MEZ

pers. com.). In the United States, OPs and CBs are recommended for pest control on a variety of crops including rice and turf grass (Fagerness et al. 2001, Merchant 2005, Way and Cockrell 2007).

As part of their annual journey between breeding and non-breeding ranges, migratory shorebirds cross international boundaries in search of available stop-over habitat. With the loss of natural wetlands and grasslands (Knopf 1994, Skagen 2006), shorebirds are forced to use human-altered habitats. Rice fields and turf grass farms provide important alternative wintering and migratory stopover habitats for shorebirds (Twedt et al. 1998, Corder 2005, Blanco et al. 2006b, Robbins 2007), but also represent potential exposure to ChE-inhibiting chemicals (Flickinger et al. 1986).

Although ChE activity has traditionally been measured by destructive sampling of brain tissue, bird populations can be effectively monitored for OP and CB exposure using non-lethal methods by measuring ChE activities in blood plasma (Hooper et al. 1989, Thompson 1991).

Acetylcholinesterase (AChE), an important enzyme in the central and peripheral nervous systems, is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh), at the nerve–nerve or nerve–effector interface. Without hydrolysis, ACh accumulates in the synapse, disrupting neurotransmission, impairing behavior and physiology, and eventually leading to death (Grue et al. 1997, Goldstein et al. 1999a). Plasma ChE activity can demonstrate exposure levels consistent with intoxication and death in subsets of a population (Hooper et al. 1989, Goldstein et al. 1999a), as well as a lack of exposure (Goldstein et al. 1999b).

Comparison of ChE activity from field samples to reference values can be used alone or in conjunction with reactivation assays. Poisoning by OPs and CBs produces similar physiological effects but reactivation assays allow for differentiation between these two types of



poisonings. Reactivation assays also address potential concerns associated with inter-species or inter-individual ChE variation (Grue 1982, Hill 1989, Fossi et al. 1996). However, reference values of ChE activity are especially important if reactivation assays cannot be used because sample volumes are too small or because OP aging results in chemically stable OP–enzyme bonds (Wilson et al. 1992).

Here, we present reference values of plasma ChE activity for apparently healthy, free-living individuals of nine shorebird species that use upland and wetland habitats. To describe ChE activity within and among shorebird species, we tested five factors that are known to affect ChE activity in other birds: interspecific variation with regard to body mass and intraspecific variation with regard to sex, body condition and date and time of capture. Our estimates of plasma ChE activity are among the first values published for shorebirds and will be useful as reference values in future toxicological studies of Nearctic-Neotropical migratory shorebirds.

## **Methods**

### ***Shorebird Capture***

Shorebird capture occurred in three states (Texas, Kansas, and Nebraska) in the United States, and three countries (Paraguay, Argentina, and Uruguay) in South America from April through December 2006. Data on body mass data were pooled from all capture sites. The subset of data used for baseline plasma ChE analysis included individuals captured between 22 April and 1 June 2006 during northbound migration in the United States at protected wetlands and grasslands. Northbound migration capture sites included Anahuac National Wildlife Refuge, Chambers County, TX (29°34'N, 94°32'W), Quivira National Wildlife Refuge, Stafford County, KS (38°08'N, 98°29'W), Konza Prairie Biological Station, Riley County, KS (39°04'N, 96°33'W) and Kissinger Wildlife Management Area, Clay County, NE (40°26'N, 98°06'W). In

2006, rice production at Anahuac National Wildlife Refuge was strictly organic, and there were restrictions on pesticide application around Quivira National Wildlife Refuge boundaries (M. Whitbeck pers. com., USEPA 2006). Konza Prairie and Kissinger Wildlife Management Area are natural preserves that were also pesticide free (E. Horne and R. Souerdyke pers. com.). Shorebirds were live-captured using mist nets, night-lighting, and drop nets, under applicable state and federal research permits.

### ***Sample Collection and Preparation***

Mass of live-captured birds was measured using a Pesola spring scale ( $\pm 1.0$  g). Wing length was measured with a wing rule ( $\pm 0.5$  mm). Total head, culmen and tarsus length were measured using vernier calipers ( $\pm 0.1$  mm). All birds were fitted with a USFWS metal band with a unique number. When possible, shorebirds were sexed in the field according to Prater et al. (1977). Upland Sandpipers (*Bartramia longicauda*) were sexed using molecular markers based on the CHD gene (Baker et al. 1999, A. E. Casey unpubl. data).

Blood was collected using a 27-gauge needle and heparinized capillary tubes (70  $\mu$ L) from the brachial vein of the wing. Total blood collected per bird ranged between two to six capillary tubes (140 – 420  $\mu$ L) and was  $<1\%$  of the bird's body mass (Gaunt et al. 1999). Blood samples were transferred to 0.5 mL screw cap cryovials, stored on wet ice in the field, and centrifuged within 8 hours to separate plasma from red blood cells. Plasma samples were stored at  $-20^{\circ}\text{C}$  for less than one month and transferred to  $-80^{\circ}\text{C}$  until laboratory analysis could be conducted. All samples were assayed within one year of collection.

### ***Laboratory Analysis***

Samples were thawed immediately before ChE activity determination. As a first step, six plasma samples from each species were pooled for characterization of optimal enzyme dilution and reagent (acetylthiocholine-iodide [AThCh] and tetraisopropyl pyrophosphoramidate [iso-OMPA]) concentrations (see Appendix B). ChE activity was determined using the method of Ellman et al. (1961) as modified by Gard and Hooper (1993) for use in a 96-well spectrophotometric plate reader (Molecular Devices, Palo Alto, CA, USA) with Softmax Pro software (Molecular Devices). Final volume of each assay was 250  $\mu$ L and contained the following components in 0.05 M Trizma buffer (pH 8.0):  $3.23 \times 10^{-4}$  M final concentration (FC) of 5,5-dithio[bis-2-nitrobenzoic acid], diluted enzyme sample, and  $1.00 \times 10^{-3}$  M FC of AThCh. To separate butyrylcholinesterase (BChE) from AChE, samples were incubated with the BChE-specific inhibitor iso-OMPA at FCs between  $1.0 \times 10^{-4}$  M and  $1.0 \times 10^{-5}$  M according to the characterization of each species. BChE was calculated as the difference between total cholinesterase (TChE) and AChE activity in the presence of iso-OMPA. All samples were run in triplicate at 25°C with the spectrophotometer set in kinetic mode. Absorbance was measured at 412 nm at 15 s intervals for 180 s with 0 s lag time. ChE activities were converted from absorbance units per min to  $\mu$ moles AThCh hydrolyzed per min (units) per mL of plasma using an extinction coefficient of  $13,600 \text{ (cm} \times \text{M)}^{-1}$ .

### ***Statistical Analysis***

All statistical analyses were conducted using procedures of SAS (ver 9.1, SAS Institute, Cary, NC, USA). All ChE activity fell within  $\pm 3$  SD of the mean except for two TChE and BChE values for Least Sandpiper which were over 4.5 times the mean for this species. These two outliers were removed from subsequent analysis. All data presented are in raw form but

statistical results are based on  $\log_{10}$ -transformed data to correct for allometric scaling. General linear models (Proc GLM) were used to determine the relationship of plasma ChEs among species using a single factor fixed effects ANOVA. Regression models (Proc REG) were calculated for plasma ChEs and time of capture, date of capture, and body condition for species with  $\geq 15$  samples. For those species where sex could be reliably determined, sex differences in plasma ChEs were compared using a Student's t-test (Proc TTEST). Time of capture was divided into four time periods of six-hour blocks each according to the following criteria:

1 = 0h – 05:59h, 2 = 06:00h – 11:59h, 3 = 12:00h – 17:59h, and 4 = 18:00h – 23:59h.

A multivariate index of body condition was computed by regressing the mass of each individual at capture on PC1 from principal components analysis (PCA), using the residuals as an index of body condition. PCA analyses were based on four morphological measurements, total head, culmen, wing, and tarsus, and were calculated separately for each species. PC1 explained between 34% and 66% of the variation in the four morphometrics. PC1 was an index of body size because all eigenvectors were positive in seven of nine species; in the remaining two species one eigenvector was negative (K. M. Strum unpubl. data). Average mass for each species was calculated using a larger dataset of captured birds that included the subset used in ChE analysis. All tests were two-tailed and considered significant at an  $\alpha$ -level = 0.05 after Bonferroni correction for the number of tests (Rice 1989).

## **Results**

During northbound migration, we captured 174 individuals from 16 shorebird species, and obtained sufficient plasma for ChE analysis from 138 individuals of 9 species. All samples were used in analysis of AChE activity and after removing the two outliers of Least Sandpiper TChE and BChE activity, 136 samples were used. We calculated average body mass for these

nine species from captures of 511 individuals at migratory and non-breeding sites throughout the Western Hemisphere. Our study species included: American Golden-Plover (*Pluvialis dominica*), Killdeer (*Charadrius vociferus*), Upland Sandpiper, Buff-breasted Sandpiper (*Tryngites subruficollis*), Pectoral Sandpiper (*Calidris melanotos*), White-rumped Sandpiper (*C. fuscicollis*), Stilt Sandpiper (*C. himantopus*), Least Sandpiper (*C. minutilla*), and Semipalmated Sandpiper (*C. pusilla*).

TChE and BChE were highly correlated ( $r^2 = 0.983$ ,  $P < 0.001$ ,  $n = 136$ ). TChE and AChE were also significantly correlated ( $r^2 = 0.533$ ,  $P < 0.001$ ,  $n = 136$ ) though less variation in TChE could be explained by AChE. Some results are reported for BChE and AChE only. TChE values for comparisons to other studies can be obtained by combining our AChE and BChE values provided that substrate, substrate concentration, and assay temperature are identical. Plasma BChE activity varied negatively with body size ( $F_{8,127} = 20.3$ ,  $P < 0.001$ ) as did AChE ( $F_{8,129} = 11.0$ ,  $P < 0.001$ ; Fig. 2-1). Mean AChE ranged from 0.24 units/mL ( $\pm 0.14$  SD,  $n = 25$ ) in Upland Sandpipers to 0.72 ( $\pm 0.38$  SD,  $n = 34$ ) in White-rumped Sandpipers, whereas mean BChE ranged from 0.83 ( $\pm 0.01$  SD,  $n = 2$ ) in American Golden-Plovers to 5.92 ( $\pm 2.85$  SD,  $n = 19$ ) in Semipalmated Sandpipers (Table 2-1). Values for Least Sandpiper outliers were BChE: 15.68 and 19.66, TChE: 16.04 and 20.17. Both of these individuals were females and had longer than average wing chord ( $\geq 100$  mm).

Sex differences in plasma ChEs were evaluated in two species, Semipalmated and Upland Sandpipers. Mean BChE was lower in male Upland Sandpipers ( $1.28 \pm 0.54$  SD,  $n = 13$ ) than females ( $1.89 \pm 0.67$  SD,  $n = 12$ ;  $t_{23} = 2.60$ ,  $P = 0.016$ ). However, mean AChE was not significantly different between male ( $0.20 \pm 0.07$  SD,  $n = 13$ ) and female ( $0.28 \pm 0.19$  SD,

n = 12) Upland Sandpipers ( $t_{14.9} = 0.50$ ,  $P = 0.615$  [unequal variance]). Similarly, mean plasma ChEs did not differ between male (AChE:  $0.55 \pm 0.25$  SD, n = 7; BChE:  $5.70 \pm 2.95$  SD, n = 7) and female Semipalmated Sandpipers (AChE:  $0.41 \pm 0.19$  SD, n = 12; BChE:  $6.04 \pm 2.92$  SD, n = 12, AChE:  $t_{17} = -1.02$ ,  $P = 0.323$ ; BChE:  $t_{17} = -0.06$ ,  $P = 0.956$ ).

In four species, the relationship between plasma ChEs and date of capture, time of capture and body condition were analyzed (Table 2-2). Three species showed trends in ChE activity as a function of capture date. Levels of BChE activity increased throughout the capture period in Upland Sandpipers ( $r^2 = 0.276$ ,  $F_{1,23} = 8.8$ ,  $P = 0.007$ ) and Least Sandpipers ( $r^2 = 0.298$ ,  $F_{1,16} = 6.79$ ,  $P = 0.019$ ). AChE activity increased throughout the capture period in White-rumped Sandpipers ( $r^2 = 0.117$ ,  $F_{1,32} = 4.24$ ,  $P = 0.048$ ). Trends were marginally significant in these three species after Bonferroni corrections for the number of tests (Rice 1989). Other plasma ChE components did not vary with capture period in any of these species. There was no significant relationship between time of capture or body condition for any species tested.

## Discussion

Interspecific variation in plasma BChE activity decreased with increasing shorebird mass similar to results from a study of plasma ChEs in European raptors (Roy et al. 2005). Mass-specific metabolic demands decrease as shorebird body size increases (Kvist and Lindström 2001), which may be a partial explanation for the inverse relationship between shorebird plasma ChE activity and body mass. Based on the high correlation between TChE and BChE, most of the variation in shorebird TChE can be attributed to BChE activity. BChE has been shown to successfully buffer AChE inhibition from some OP chemicals (Leopold 1996, Parker and Goldstein 2000). Birds lack A-esterases which hydrolyze OP and CB pesticides (Aldridge 1953)

and higher levels of BChE activity may provide some protection against poisoning and information about exposure.

Inclusion of all ChE activity results is important when presenting baseline ChE values, however extreme outliers may influence statistical tests. For this reason, we removed two outliers from our dataset before analysis. The causes of extreme BChE activity were unknown but the two individuals with outlier values could have had liver damage or unusual levels of fat metabolism during migration (Rattner and Fairbrother 1991, Valle et al. 2006).

Due to the interspecific variation in ChE activity with regard to body size, our data can be used to estimate normal plasma ChE activity levels of species without reference values for field sample comparison. While there is no substitute for species-specific reference values, patterns of mass-specific variation in plasma ChE activity provide an initial framework for assessing exposure in other shorebird species.

We found sex differences in mean plasma BChE in one species, the Upland Sandpiper. At the time of capture, females were heavier than males (female mean mass = 166 g, n = 12; male mean mass = 136 g, n = 13) and would be expected to have lower plasma ChE activities based on the interspecific results of this study. However, females of Upland Sandpiper had higher mean BChE activity than males. Our results may be related to breeding condition because Upland Sandpipers evaluated in this study had recently arrived on the breeding grounds and many females were at an egg-laying stage (B. K. Sandercock unpubl. data). An increase in plasma ChEs during egg-laying has been reported in other avian species (Rattner and Fairbrother 1991). Samples of Upland Sandpiper during the non-breeding season as well as samples from males and females of other shorebird species on the breeding grounds are needed to further investigate this idea.

The condition of individual shorebirds was not related to plasma ChE activity in our study. This is an important result since the physiological stress of migration can result in inter-individual variation in body condition depending on the time since arrival at a stopover site and the distance traveled prior to capture. Individuals in better condition presumably have more fat and muscle translating into larger relative mass (Schulte-Hostedde et al. 2005), unrelated to ChE activities. However, birds that died from anti-ChE exposure had lower fat and muscle scores due to reduced food intake after poisoning (Grue 1982). In our study, body condition was not used as an indicator of chemical exposure as it might be in another study.

Increases in plasma ChEs were marginally significant in three species throughout the capture season. In each case, the percent of variation explained in plasma ChE activity was fairly low ( $r^2 < 0.3$ ). Seasonal variation in mean plasma ChEs has been detected in other migratory birds and has been attributed to changes in diet (Goldstein et al. 1999b). In shorebirds, variation in plasma ChEs during the capture season could be due to changes in diet or to changes in physiological condition caused by changes in organ size during migration (Piersma and Gill 1998). Further investigation of the relationship between ChEs and date will be conducted using data from individuals sampled in South America. With larger datasets from additional sites, seasonal patterns in ChE activity may be more apparent.

The new data presented here provide a starting point for understanding variation in plasma ChEs in Nearctic-Neotropical migratory shorebirds. Future analyses should be conducted with samples collected at non-breeding sites in South America as well as the breeding grounds. The relationship of plasma ChEs to environmental covariates should be further explored to provide a more complete picture of shorebird plasma ChEs throughout the annual cycle. Data on ChE activity could then be used to assess shorebird exposure to ChE-inhibiting



pesticides at any time of year. Once exposure is determined, efforts could be focused on affected species to evaluate if the level of exposure poses a population threat. If so, efforts could begin on developing regulations for OP and CB pesticides through partnerships with local and international governments. If future studies demonstrate that shorebird exposure to ChE-inhibitors is limited, this information will be used to redirect research efforts into other possible causes of shorebird population declines.

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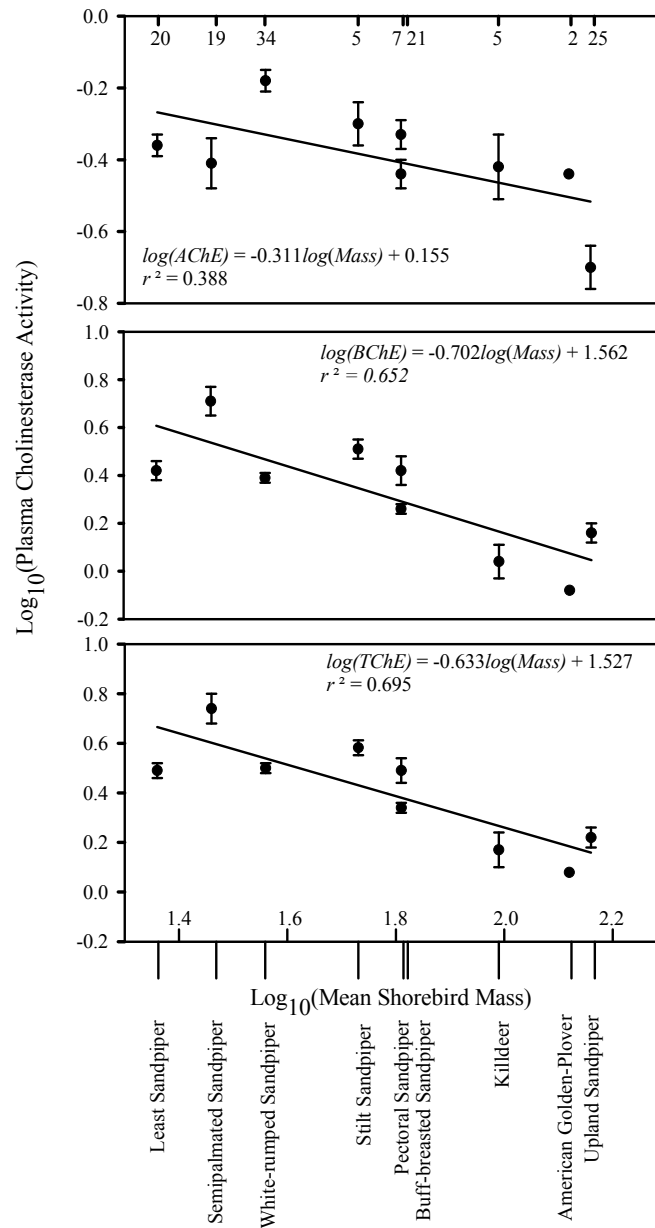
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**Figure 2-1 Log<sub>10</sub>-log<sub>10</sub> graph showing the relationship between mean body mass and ChE activity in 9 species of shorebirds captured during spring migration in the Great Plains region of the United States. Sample sizes are inside the uppermost x-axis and error bars represent  $\pm$  SE. Pectoral Sandpiper (*Calidris melanotos*) is the higher of the two log<sub>10</sub>(ChE) values where log<sub>10</sub>(body mass) = 1.81.**



**Table 2-1 Descriptive statistics of ChE activity (units/mL plasma) for 9 shorebird species sampled during northbound migration in the Great Plains region of the United States including sample size of individuals (n)<sup>†</sup>, mean, standard deviation (SD), minimum (min) and maximum (max) values.**

Species	n	TChE				AChE				BChE			
		mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
American Golden-Plover	2	1.20	0.01	1.20	1.21	0.37	0.00	0.37	0.37	0.83	0.01	0.83	0.84
Killdeer	5	1.56	0.55	0.91	2.42	0.41	0.16	0.21	0.59	1.15	0.45	0.69	1.89
Upland Sandpiper	25	1.81	0.70	0.69	3.29	0.24	0.14	0.02	0.65	1.57	0.67	0.51	3.11
Buff-breasted Sandpiper	21	2.18	0.38	1.71	3.11	0.35	0.11	0.16	0.65	1.79	0.27	1.43	2.24
Pectoral Sandpiper	7	3.22	0.80	1.53	4.01	0.48	0.12	0.32	0.66	2.74	0.72	1.21	3.35
White-rumped Sandpiper	34	3.23	0.80	1.95	4.75	0.72	0.38	0.30	2.31	2.56	0.69	1.53	3.99
Stilt Sandpiper	5	3.82	0.67	2.89	4.72	0.52	0.16	0.37	0.69	3.31	0.60	2.45	4.03
Least Sandpiper	20	3.37	0.81	1.92	4.67	0.45	0.10	0.32	0.60	2.92	0.76	1.33	4.16
Semipalmated Sandpiper	19	6.38	2.95	1.28	10.78	0.46	0.22	0.07	0.89	5.92	2.85	1.21	10.11

<sup>†</sup>Total sample size. Estimates of TChE and BChE exclude two outliers of Least Sandpiper.

**Table 2-2 Trends in plasma ChE activity of 5 species of shorebirds as a function of date of capture, time of capture and an index of body condition using  $\log_{10}$  transformed ChE activity. After sequential Bonferroni correction for number of tests, test statistics were considered significant at an  $\alpha$ -level of 0.05 if  $P < 0.002$ .**

Species	ChE Type	Date of Capture			Time of Capture			Index of Body Condition		
		df	<i>F</i>	$P \leq$	df	<i>F</i>	$P \leq$	df	<i>F</i>	$P \leq$
Upland Sandpiper	log(AChE)	1,23	0.0	0.956	1,23	0.9	0.359	1,23	1.8	0.193
	log(BChE)	1,23	8.8	0.007	1,23	0.0	0.836	1,23	0.2	0.640
Buff-breasted Sandpiper	log(AChE)	1,19	0.0	0.891	1,19	0.3	0.858	1,19	0.2	0.692
	log(BChE)	1,19	2.2	0.152	1,19	0.8	0.373	1,19	0.4	0.550
White-rumped Sandpiper	log(AChE)	1,32	4.2	0.048	1,32	0.1	0.750	1,32	0.0	0.876
	log(BChE)	1,32	2.5	0.123	1,32	0.2	0.653	1,32	0.3	0.599
Least Sandpiper	log(AChE)	1,18	0.1	0.820	1,16	0.6	0.452	1,18	0.5	0.498
	log(BChE)	1,16	6.8	0.019	1,14	0.0	0.886	1,16	0.0	0.977
Semipalmated Sandpiper	log(AChE)	1,17	0.1	0.736	1,17	0.3	0.615	1,16	0.0	0.926
	log(BChE)	1,17	3.1	0.097	1,17	0.1	0.715	1,16	0.7	0.419



## **CHAPTER 3 - Exposure of migratory shorebirds to cholinesterase-inhibiting contaminants in the Western Hemisphere**

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### **Abstract**

Migratory shorebirds traverse long distances during their annual movements and access to quality habitats is critical to completion of a successful migration. Many natural habitats have been degraded or destroyed and shorebirds increasingly utilize agricultural habitats. Use of cultivated fields and similar habitats may increase risk of exposure to cholinesterase (ChE)-inhibiting pesticides. To evaluate exposure of migratory shorebirds to organophosphorus (OP) and carbamate (CB) pesticides, we sampled birds at stopover sites during spring and fall migration in North America and at non-breeding sites in South America. Birds were sampled from reference sites with no known OP or CB applications and pesticide use sites where agrochemicals were recommended for control of crop pests. Blood samples and footwashes were collected from live-captured individuals. Plasma acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity levels of Buff-breasted Sandpipers were lower at pesticide use sites in South America but BChE activity was higher at pesticide use sites in North America. There were no differences between reference sites and pesticide use sites in four other species tested. Regional differences in plasma ChE activities from reference sites were tested in two species. Plasma AChE activity was higher among White-rumped Sandpipers sampled in North America but there was no difference between regions for Buff-breasted Sandpipers. A meta-analysis across all species indicated 4 of 6 effect sizes were negative for AChE but there was an

overall positive effect of pesticide use sites on BChE activity in North America. Comparisons of body mass between sites and regions suggest that habitat use did not affect maintenance of body mass. Our study provides the first prospective estimates of shorebird exposure to ChE-inhibiting pesticides. Overall, our results suggest that 1 of 5 shorebird species tested was exposed to ChE-inhibiting pesticides at the non-breeding grounds and continued monitoring is necessary. We highlight the importance of complete sampling and addressing variability in plasma ChEs as part of evaluating potential exposure to organophosphorus and carbamate pesticides.

## **Introduction**

Ongoing declines in populations of migratory shorebirds have spurred research to investigate possible environmental factors driving population dynamics range-wide. Exposure to environmental contaminants, habitat loss and degradation, disturbance, and illegal hunting have been suggested as anthropogenic factors that may limit shorebird populations (Senner and Howe 1984, Page and Gill 1994, Zöckler et al. 2003). Previous research on shorebird exposure to environmental contaminants has focused on heavy metals, organochlorines, and polychlorinated biphenyls (White et al. 1980, White et al. 1983, Custer and Myers 1990, Custer and Mitchell 1991, Burger et al. 1993, Hui 1998, Hui et al. 2001, McFarland et al. 2002). Few studies have investigated exposure of migratory shorebirds to pesticides that act by cholinesterase (ChE) inhibition (Mitchell and White 1982, Fair et al. 1995, Iko et al. 2003). These chemicals are potent insecticides used to control agricultural pests throughout North and South America. For example, over 49 million kg of insecticides were applied to protect crops in the United States in 2001. Of these, 70% were ChE-inhibiting organophosphorus (OP) and carbamate (CB) pesticides (Kiely et al. 2004). These two types of pesticides replaced organochlorines due to their relatively rapid degradation and lack of bioaccumulation. Nevertheless, OPs and CBs are

highly toxic over the short-term and may pose a risk to non-target, avian species. Migratory birds have been poisoned while exploiting resources in agricultural habitats in South America (Basili and Temple 1995, Goldstein et al. 1999a). In addition to mortality, sub-lethal exposure to OPs and CBs can cause a suite of physiological impairments including loss of migratory orientation and decreased flight speed (Vyas et al. 1995, Grue et al. 1997, Brasel et al. 2005).

The Great Plains region of the United States is an important flyway for migratory shorebirds that breed at arctic or north temperate latitudes and winter in Southern South America (Skagen et al. 1999). The landscape of the Great Plains flyway is dominated by agriculture. Similarly, non-breeding habitats in South America have largely been converted into agricultural and rangeland areas (Wetmore 1927, Soriano et al. 1992, Isacch and Martínez 2003, Blanco et al. 2006). Thus, evaluation of potential exposure to ChE-inhibiting pesticides is important to conservation efforts of migratory shorebirds.

Life history traits and migratory pathways of shorebirds may determine risk of pesticide exposure for different species. Nearctic-Neotropical shorebird migrants can be categorized into two groups based on their habitat requirements: upland and wetland. Upland shorebirds prefer drier habitats comprised of low vegetation and include American Golden-Plover (*Pluvialis dominica*), Upland Sandpiper (*Bartramia longicauda*), and Buff-breasted Sandpiper (*Tryngites subruficollis*; Myers and Myers 1979, Isacch and Martínez 2003). These three species regularly use agricultural habitats at stopover and wintering sites where they may come into direct contact with OPs and CBs. Upland shorebirds consume a variety of invertebrates, including agricultural pests, whose population numbers increase during shorebird migration (Houston and Bowen 2001, Nagoshi and Meagher 2004, Isacch et al. 2005). Wetland species, such as Least Sandpiper (*Calidris minutilla*), Pectoral Sandpiper (*C. melanotos*), and White-rumped Sandpiper

(*C. fuscicollis*) require habitats with standing water, and frequently use rice fields and other agricultural areas where use of ChE inhibitors may be widespread (Hands et al. 1991, Skagen and Knopf 1993, Twedt et al. 1998, Skagen et al. 2005, Blanco et al. 2006). In fact, small numbers of shorebirds have been found dead in rice fields after the application of carbofuran, a potent anti-ChE (Flickinger et al. 1980; 1986, Littrell 1998).

ChE activity in brain or plasma is often measured as an indication of exposure to OP and CB pesticides (Soler-Rodriguez et al. 1998). Measurement of brain ChE activity is useful for diagnosing OP or CB poisoning post-mortem but requires euthanizing the bird. On the other hand, plasma ChE activity is sensitive to low dose exposure to OPs and CBs and can be used to monitor sub-lethal exposure among live-captured individuals (Hooper et al. 1989, Thompson 1991, Wilson et al. 1991, Soler-Rodriguez et al. 1998). There are two main types of ChE in avian plasma. Acetylcholinesterase (AChE) is responsible for hydrolysis of the neurotransmitter acetylcholine and maintains proper functioning of the central and peripheral nervous systems. The exact function of butyrylcholinesterase (BChE) is unknown although it has been shown to successfully buffer AChE inhibition from some OP compounds (Leopold 1996, Parker and Goldstein 2000).

Both enzymes can be used to monitor exposure to OP and CB pesticides by comparison of field samples to reference values or by reactivation of inhibited enzyme (Hooper et al. 1989, Strum et al. 2008). Plasma ChEs can vary on spatial and temporal scales (Goldstein et al. 1999b) but reference values rarely control for these factors. Similarly, reactivation attempts can be hampered by dealkylation and spontaneous reactivation of inhibited enzymes (Wilson et al. 1992). Evaluating OP and CB exposure in migrant birds must account for sources of variability in reference values that can interfere with transcontinental ChE monitoring efforts.

The purpose of our study was to evaluate exposure of migratory shorebirds to ChE-inhibiting pesticides. We sampled shorebirds at stopover sites in North America and at their non-breeding grounds in South America. In previous work, we reported reference values of plasma ChE activity for nine species of migratory shorebirds sampled at stopover sites in the Great Plains and addressed variability of plasma ChEs (Strum et al. 2008). Here, we provide the first values for non-breeding shorebirds in South America. Moreover, we compared plasma ChE activity and body mass between sites with no OP and CB use versus sites where ChE-inhibitors were recommended for pest control. We calculated an effect size of habitat type on ChE activity across all species. We also tested for variation in plasma ChEs by region to assess the need for reference values at different temporal and spatial scales. If shorebirds were exposed to pesticides, we predicted plasma ChE activity and mean body mass would be lower in samples from sites with insecticide use (Grue et al. 1997, Goldstein et al. 1999b). We predicted a change in ChE by region if dietary changes were occurring between the breeding and non-breeding grounds (Goldstein et al. 1999b). Our study represents the first large-scale, prospective evaluation of anti-ChE pesticide exposure to address a potential conservation threat to migratory shorebirds throughout their range (Hooper et al. 2003).

## **Methods**

### ***Shorebird Capture***

We captured shorebirds in three states (Texas, Kansas and Nebraska) in the United States from April to August 2006 and March to June 2007, and three countries (Argentina, Paraguay, and Uruguay) in South America from September to December 2007. We concentrated our capture efforts at natural sites with no known pesticide use (reference sites) and agricultural sites

where OPs and CBs were recommended for pest control according to national or state crop guidelines (pesticide use sites).

During northbound migration in 2006, four reference sites in North America included coastal prairie and organic rice fields at Anahuac National Wildlife Refuge, Chambers County, TX (29°34'N, 94°32'W), salt marshes at Quivira National Wildlife Refuge, Stafford County, KS (38°08'N, 98°29'W), native grassland at Konza Prairie Biological Station, Riley County, KS (39°04'N, 96°33'W), and wetlands at Kissinger Wildlife Management Area, Clay County, NE (40°26'N, 98°06'W). Rice production at Anahuac National Wildlife Refuge was strictly organic and there were restrictions on application of pesticides around Quivira National Wildlife Refuge boundaries (M. Whitbeck pers. com., USEPA 2006). Konza Prairie and Kissinger Wildlife Management Area are natural reserves that were also pesticide free (E. Horne and R. Souerdyke pers. com.). During southbound migration in 2006 and northbound migration in 2007, shorebirds were sampled at one reference site, salt marshes of Bolivar Flats Shorebird Sanctuary (29°78'N, 94°77'W) and three agricultural sites, turf grass farms near Lawrence, KS (38°58'N, 95°14'W), Colwich, KS (37°46'N, 97°32'W), and Crosby, TX (29°54'N, 95°03'W).

Shorebirds were captured during the non-breeding season in 2006 in Southern South America. Three reference sites included inundated grasslands along the shoreline of Bahía de Asunción, Paraguay (25°16'S, 57°37'W), salt marshes and shoreline of Laguna Mar Chiquita, Cordoba, Argentina (30°32'S, 62°17'W), and coastal grasslands of Laguna de Rocha, Rocha Department, Uruguay (34°40'S, 54°17'W). Bahía de Asunción and Laguna Mar Chiquita are designated as sites of regional and hemispheric importance for shorebirds by the Western Hemisphere Shorebird Reserve Network. Bahía de Asunción is centrally located within the city of Asunción with no adjacent agriculture. Laguna Mar Chiquita and Laguna de Rocha are

nationally protected parks, neither of which had OPs or CBs applied to local capture areas during the field season (H. Caimarys and E. Martin pers. com.). Two agricultural sites sampled in South America included rice fields and cattle pastures near San Joaquin, Santa Fe Province, Argentina (30°42'S, 60°52'W) and Coronilla, Rocha Department, Uruguay (33°48'S, 53°39'W).

Shorebirds were live-captured using mist nets, drop nets, and night-lighting under applicable state, federal, and international research permits. In addition, a small number of Buff-breasted Sandpipers were collected by shotgun at two sites, turf grass farms near Lawrence and Colwich, KS. After sampling, all birds were deposited as study skins at the University of Kansas, Museum of Natural History (Robbins 2007).

### ***Sample Collection and Preparation***

To examine body size and condition of shorebirds among sites, seven morphological measurements were recorded for each individual. Mass of live-captured birds was measured using a Pesola spring scale ( $\pm 1$  g). Wing length was measured with a wing rule ( $\pm 0.5$  mm). Total head, culmen and tarsus length were measured using vernier calipers ( $\pm 0.1$  mm). Fat deposits in the furcula, sides, and abdomen were visually scored on a 9-point scale from zero to eight, with zero indicating no fat and eight indicating large fat depots (Bairlein 1995). If possible, shorebirds were sexed in the field according to Prater et al. (1977). Upland Sandpipers and Buff-breasted Sandpipers were sexed using molecular markers based on the CHD gene (Baker et al. 1999, A.E. Casey unpubl. data, K. M. Strum unpubl. data.). All birds were fitted with a USFWS metal band with a unique number.

Field samples for pesticide exposure included blood, brain tissue, and footwashes. Blood was collected using a 27-gauge needle and heparinized capillary tubes (70  $\mu$ L) from the brachial vein of the wing. Total blood collected per bird ranged between two to six capillary tubes

(140 to 420  $\mu$ L) and was <1% of the bird's body mass (Gaunt et al. 1999). Blood samples were transferred to 0.5 mL screw cap cryovials, stored on wet ice in the field, and centrifuged within 8 hours to separate plasma from red blood cells. Plasma samples were stored at -20°C for one to four weeks and transferred to -80°C until laboratory analyses could be conducted. Collected individuals were wrapped in chemically-clean foil, placed on wet ice in the field and frozen at -80°C within 8 hours. Brains were excised partially-frozen, placed into 2.0 mL Eppendorf tubes and immediately refrozen at -80°C. The tarsi and feet of captured and collected individuals were rinsed with 15 mL pesticide grade 2-propanol collected in an amber glass jar with a Teflon lined lid via a stainless steel funnel. Footwashes were stored on wet ice in the field and transferred to -20°C until analyses could be conducted. All samples were analyzed within one year of collection.

### ***ChE Activity***

Laboratory analyses of ChE activity were conducted at The Institute for Environmental and Human Health (TIEHH) at Texas Tech University and at the Division of Biology at Kansas State University (KSU). Samples analyzed at TIEHH were read on a SpectraMax plate reader (Molecular Devices, Palo Alto, CA, USA) with Softmax Pro software (Molecular Devices). KSU samples were analyzed in a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) with Microplate Manager software (ver 5.2.1, Bio-Rad Laboratories). Assay conditions and reagent concentrations were identical between labs to minimize inter-lab differences. To quantify within and between lab variation, aliquots of horse serum (Invitrogen Corporation, Carlsbad, CA, USA) of known ChE activity were run on each plate containing shorebird samples (see Appendix A).



Sample preparation differed according to tissue type. Plasma samples were thawed immediately before ChE activity determination and diluted to a species-specific concentration with cold 0.5 M, 7.4 pH buffer. Brains were thawed and homogenized using the Tissue Miser electric homogenization tool (Fisher Scientific, Pittsburgh, PA, USA) at a 1:3 ratio of tissue:buffer. ChE activity was determined immediately after homogenization on a 60- or 72-fold serially diluted sample.

All ChE activity for plasma and brain was determined using the method of Ellman et al. (1961) as modified by Gard and Hooper (1993) for use on a 96-well plate reader. Final volume of each assay was 250  $\mu$ L and contained the following components in 0.05 M Trizma buffer (pH 8.0):  $3.23 \times 10^{-4}$  M final concentration (FC) of 5,5-dithio[bis-2-nitrobenzoic acid], diluted enzyme sample, and  $1.00 \times 10^{-3}$  M FC of acetylthiocholine-iodide (AThCh). To separate activity of BChE from AChE, a portion of each sample was incubated with the BChE-specific inhibitor, tetraisopropyl pyrophosphoramidate (iso-OMPA), at FC  $1.0 \times 10^{-4}$  M. BChE activity was calculated as the difference between total cholinesterase (TChE) measured without iso-OMPA and AChE activity in the presence of iso-OMPA. All samples were run in triplicate at 25°C with the spectrophotometer set in kinetic mode. Absorbance was measured at 412 nm at 15 s intervals for 180 s with a lag time of 0 s. ChE activities were converted from absorbance units per min to  $\mu$ moles AThCh hydrolyzed per min (units) using an extinction coefficient of  $13,600 \text{ (cm} \times \text{M)}^{-1}$  and were expressed as units per mL of plasma or units per g of brain tissue.

### ***Reactivation Assays***

Mean plasma ChE activity was lower at pesticide use sites than at reference sites in one species, the Buff-breasted Sandpiper (see Results). Reactivation assays were conducted to test for OP and CB inhibition of enzyme activity on samples from this species with an adequate

volume after dilution ( $\geq 1.3$  mL). Reactivation of OP-inhibited enzyme required two aliquots of diluted sample. One aliquot was incubated with  $1.00 \times 10^{-4}$  M FC pyridine-2-aldoxime methochlorine (2-PAM) at 25°C to induce chemical reactivation. The remaining aliquot received an equal volume of distilled water and was incubated alongside the sample with 2-PAM as a negative control. Aliquots were assayed after 0.5 hr. CB-inhibited enzyme reactivation analysis also required two additional sample aliquots. One aliquot was incubated at 37°C to induce spontaneous reactivation whereas the other aliquot was maintained on ice as a negative control. Both aliquots were assayed after one hr.

### ***Chemical Residue Analyses***

Footwash samples were screened against reference standards for 13 OPs and 7 CBs at a detection limit of 0.5 ug/mL or 1 ug. OP residues were analyzed on a HP 6890 gas chromatograph (GC) equipped with a nitrogen-phosphorous detector (NPD). The column (J & W Scientific, Rancho Cordova, CA, USA) was a 30-m x 0.25 mm i.d. fused silica capillary coated with a DB-1701 stationary phase (film thickness of 0.25  $\mu$ m) and a carrier gas (He) flow rate of 1.5 mL/min. Injector and detector temperatures were 250°C and 275°C, respectively. CB residues were analyzed on a HP 1100 Series liquid chromatograph (HPLC) with a Post-Column Derivatizer (PCX) 5200<sup>®</sup> (Mountain View, CA, USA) and a fluorescence detector (excitation and emission wavelengths of 330 nm and 465 nm, respectively). The column (Discovery<sup>®</sup> C<sub>8</sub>, Supelco, St. Louis, MO, USA) was 250 mm x 4.6 mm (5  $\mu$ m particle size) operated at a flow rate of 0.8 mL/min. Standard operating conditions were a water:methanol ratio of 88:12 for 2 min, followed by an increase in methanol to 34:66 over 40 min with hold time of 4 min followed by an increase to 100% methanol in 0.1 min with a final hold time of 3 min.

For both GC and HPLC analyses, an internal standard adjusted for some of the internal variation of the instrument, while an intermittent standard was run after every 5 injections to assess variability. If the intermittent standard varied by more than 10%, the sample set was re-run. A five-point standard curve was constructed from constant volume injections of calibration standards. Computer-generated peak areas were used to measure sample concentrations in an external standard method.

### ***Statistical Analyses***

Statistical analyses were performed with Program SAS (ver 9.1.3, SAS Institute, Cary, NC, USA). We performed our meta-analysis with Program MetaWin (ver 2.0, Sinauer Associates, Sunderland, MA, USA). An important covariate for variation in plasma ChE activity is interspecific variation in body size and we conducted analyses separately for each species of shorebird (Roy et al. 2005, Strum et al. 2008).

Mean ChE activity averaged from triplicate readings was used in analyses if the coefficient of variation was below 10%. Z-tests were used to identify outliers where observations were  $\geq 3SD$  of the overall mean for each species. Comparisons of mean ChE activities between regions (North vs. South America) and between reference sites and pesticide use sites within regions were performed using t-tests (Proc TTEST). Tests for differences between regions and between reference sites and pesticide use sites were two-tailed. Tests for differences between regions were calculated using data from reference sites only.

Despite considerable sampling effort, we were unable to capture all species at each combination of region and site, which precluded use of standard statistical analysis with factorial models. Thus, to synthesize the effect of habitat (reference vs. pesticide use) on ChE activity among shorebirds, we calculated individual, regional, and cumulative effect sizes, using

Hedges'  $d$  as a measure of effect size (Gurevitch and Hedges 1993). Our meta-analysis quantified an overall effect of treatment (pesticide use sites) on a response variable (ChE activity). The magnitude of the effect size ( $d$ ) is unbounded and can be positive or negative. According to Cohen (1988), values of  $d \leq 0.2$  are small,  $d = 0.5$  are moderate, and  $d \geq 0.8$  indicates a large effect. We used reference sites as the control and negative effect sizes indicate that individuals captured at pesticide use sites had lower ChE activity than birds sampled at reference sites.

ChE reactivation was evaluated using a two-tailed t-test (Proc TTEST). To evaluate reactivation of OP-inhibited enzyme, we compared the post-incubation ChE activity of the sample incubated with 2-PAM versus the negative control. Similarly, CB inhibition of enzyme was tested by comparing post-incubation activity of the incubated sample versus the sample kept on ice. Some sample volumes were insufficient to conduct all reactivation analyses resulting in unequal sample sizes between assays.

One potential effect of ChE inhibition can be reduced feeding and anorexia (Grue 1982, Grue et al. 1997). To test for a physiological effect of ChE inhibition, we first examined whether fat score was correlated with body mass. We then used body mass as an indicator of fitness and compared mean body mass between regions and treatments (sites) within a region using a two sample t-test (Proc TTEST). Comparisons between regions were conducted using data from reference sites only.

## **Results**

We sampled a total of 671 individuals of 14 shorebird species at stopover sites and non-breeding sites in the Western Hemisphere. Our study species included 11 species of latitudinal

migrants and 3 species of Neotropical endemics. Number of individuals of a species captured ranged from 5 (South American Painted-Snipe) to 122 (White-rumped Sandpiper; Table 3-1).

Of the captured individuals, 452 birds had sufficient sample volumes of plasma for analyses of ChE activity. Two Least Sandpipers had BChE activity 15 to 20 SD greater than the mean for this species as reported in Strum et al. (2008) and were omitted from statistical analyses. In South America, mean AChE (units/mL plasma) ranged from a low of 0.28 (Buff-breasted Sandpiper) to 0.61 (Lesser Yellowlegs) and mean BChE activity ranged from 0.58 (South American Painted-Snipe) to 5.38 (Lesser Yellowlegs; Table 3-2). In North America, we found that mean AChE ranged from a low of 0.27 (Upland Sandpiper) to 0.73 (White-rumped Sandpiper) and mean BChE activity ranged from a low of 1.63 (Upland Sandpiper) to 8.34 (Semipalmated Sandpiper; Table 3-3).

### ***Spatial Variation in Plasma ChE Activity***

Variation in plasma ChEs by site (reference vs. pesticide use) was apparent in 1 of 5 species tested. Buff-breasted Sandpiper AChE and BChE activity was 36.7% ( $t_{21} = -2.26$ ,  $P = 0.034$ ) and 31.6% lower ( $t_{21} = -2.91$ ,  $P = 0.008$ ) at pesticide use sites in South America (Table 3-2, Fig. 3-1). However, Buff-breasted Sandpiper BChE activity was 21.0% higher in individuals sampled from pesticide use sites in North America ( $t_{39} = 3.47$ ,  $P \leq 0.001$ ; Table 3-3, Fig. 3-1). There were no further significant differences in ChE activity as a function of habitat-specific pesticide use in the other shorebird species with sufficient sample numbers for comparison (Tables 3-2 and 3-3, Fig. 3-1).

AChE and BChE activity from reference sites differed between regions for 1 of 2 species (Tables 3-2 and 3-3, Fig. 3-2). White-rumped Sandpiper AChE was 19.3% higher in individuals sampled in North America than South America ( $t_{97} = 2.50$ ,  $P = 0.014$ ). Similarly, mean BChE

activity was 26.7% higher for Pectoral Sandpipers sampled in North America but statistical inferences were not possible due to the small sample size from South America. There was no significant difference in mean plasma AChE or BChE activity between regions in Buff-breasted Sandpipers sampled at reference sites.

Mean TChE activity of Buff-breasted Sandpiper brains collected at turf grass farms was 24.3 units/g brain ( $\pm 3.1$  SD,  $n = 6$ ; min: 18.5, max: 27.5). Plasma TChE of Buff-breasted Sandpipers sampled at pesticide use sites in North America was 2.7 units/mL plasma ( $\pm 0.6$  SD,  $n = 20$ ) and represented 10.9% of brain TChE activity.

The effect size of habitat on ChE activity was variable among species and between regions and overall, effect sizes were non-significant for AChE ( $d = -0.15$ , 95% CI: -0.41 to +0.10) and BChE ( $d = 0.18$ , 95% CI: -0.35 to +0.60; Tables 3-2 and 3-3). Species-specific effect sizes for AChE were negative for 4 of 6 comparisons and ranged from a low of -0.96 in Buff-breasted Sandpipers to +0.27 in American Golden-Plovers, both sampled in South America. Effect sizes for BChE were negative for 1 of 6 comparisons and ranged from -1.21 in Buff-breasted Sandpipers captured in South America to +1.05 in Buff-breasted Sandpipers captured in North America. There was a significant, positive effect of site on BChE activity in North America ( $d = +0.58$ , 95% CI: +0.20 to +1.05) but not in South America ( $d = -0.11$ , 95% CI: -1.21 to +0.19). There were no significant regional effects on AChE activity (NA:  $d = -0.20$ , 95% CI: -0.29 to +0.10; SA:  $d = -0.11$ , 95% CI: -0.47 to +0.27). Based on the  $Q_w$  statistic for heterogeneity, there was variation in BChE effect sizes among species in South America ( $Q_w = 6.64$ ,  $P = 0.04$ ) but not in North America ( $Q_w = 3.52$ ,  $P = 0.17$ ). There were no differences in effect sizes among species for AChE in North or South America (NA:  $Q_w = 0.51$ ,  $P = 0.78$ ; SA:  $Q_w = 4.93$ ,  $P = 0.085$ ).

Plasma ChE reactivations were attempted in samples collected from Buff-breasted Sandpipers in South America. Not all samples had adequate volume for reactivation analyses and 12 samples from reference sites and 5 samples from pesticide use sites were tested for reactivation of plasma ChE activity. Plasma AChE and BChE of Buff-breasted Sandpipers did not reactivate using the 2-PAM reactivation method in samples from pesticide use sites (AChE:  $t_8 = -0.07$ ,  $P = 0.95$ ; BChE:  $t_8 = 0.02$ ,  $P = 0.99$ ) or reference sites (AChE:  $t_{22} = 0.33$ ,  $P = 0.74$ ; BChE:  $t_{22} = 0.45$ ,  $P = 0.66$ ). Likewise, plasma ChEs did not reactivate via spontaneous reactivation for CB-inhibition from pesticide use sites (AChE:  $t_8 = -0.03$ ,  $P = 0.97$ ; BChE:  $t_8 = -0.43$ ,  $P = 0.68$ ) or reference sites (AChE:  $t_{20} = -0.37$ ,  $P = 0.72$ ; BChE:  $t_{20} = -9.26$ ,  $P \leq 0.001$ ). No chemical residues were found in any footwash extraction.

### ***Spatial Variation in Body Mass***

Linear regression analyses indicated that fat score was a function of body mass in 7 of 13 species ( $r^2 \geq 0.4$ ,  $P \leq 0.05$ ). Thus, we used only body mass as an indicator of individual quality. There was sufficient data for regional and site comparisons in 7 species. Two of four species showed site differences in body mass in South America and all three species tested showed differences in North America (Table 3-4). In all but one species, mean body mass was higher at pesticide use sites than at reference sites. In South America, Buff-breasted Sandpipers were 9.7% heavier ( $t_{22} = 2.46$ ,  $P = 0.023$ ) and White-rumped Sandpipers were 5.7% heavier at pesticide use sites ( $t_{84} = 2.27$ ,  $P = 0.026$ ) while American Golden-Plovers ( $t_{45} = 1.39$ ,  $P = 0.17$ ) and Common Plovers ( $t_{14} = -0.11$ ,  $P = 0.91$ ) showed no difference in mean body mass between sites of capture. In the Northern Hemisphere, Buff-breasted Sandpipers were 9.9% heavier ( $t_{62} = 2.41$ ,  $P = 0.019$ ), Pectoral Sandpipers were 30.5% heavier ( $t_{35} = 4.21$ ,  $P \leq 0.001$ ), and Upland Sandpipers were 17.2% lighter ( $t_{65} = 1.91$ ,  $P = 0.06$ ) at pesticide use sites.

Two of three species tested showed regional differences in mean body mass; in all cases, body mass was greater for individuals sampled in North America (Table 3-4). White-rumped Sandpipers were 6.6% heavier ( $t_{104} = 3.16$ ,  $P = 0.002$ ) and Buff-breasted Sandpipers were 13.1% heavier ( $t_{35} = 3.49$ ,  $P \leq 0.001$ ) in North America. Stilt Sandpiper body mass did not differ between regions ( $t_{16} = 0.11$ ,  $P = 0.91$ ).

## Discussion

We conducted intensive sampling of 14 species of migratory shorebirds across the Western Hemisphere to evaluate potential exposure to ChE-inhibiting pesticides. We used plasma ChEs to evaluate exposure to organophosphorus (OP) and carbamate (CB) pesticides because the sampling procedure was non-lethal and is sensitive to most OPs and CBs (Soler-Rodriguez et al. 1998). Our results demonstrate that of three species with sufficient data for comparisons between reference sites and pesticide use sites, one Nearctic-Neotropical migratory shorebird, the Buff-breasted Sandpiper, was likely exposed to ChE-inhibiting pesticides at non-breeding sites in South America and that two other species, American Golden-Plovers and White-rumped Sandpipers, were likely not. Of three species captured during migration in North America, no evidence of exposure was detected. A meta-analysis indicated negative effects of habitat (site) use on AChE activity in 4 of 6 comparisons and on BChE activity in one species. However, there was a significant positive effect of habitat use on BChE in North America. No evidence of negative physiological effects due to ChE-inhibitor exposure or habitat use was detected using body mass.



### ***Spatial Variation in Plasma ChE Activity***

Plasma ChEs differed by site (reference vs. pesticide use) only in the Buff-breasted Sandpiper. In South America, mean plasma AChE and BChE activities of Buff-breasted Sandpipers were lower at pesticide use sites than reference sites and the magnitude of the differences was consistent with inhibition by ChE-inhibitors reported in other migratory birds (Goldstein et al. 1999a). In contrast with this result, BChE activity of Buff-breasted Sandpipers sampled at pesticide use sites in North America was 21% higher than individuals sampled at reference sites. This is opposite to the expected pattern if individuals were exposed to ChE inhibitors but is consistent with differences in total plasma ChE activity found in Mountain Plovers (*Charadrius montanus*) at an agricultural site compared to a reference site in North America (Iko et al. 2003).

We found negative effect sizes on AChE activity in 2 of 3 species in each region ranging from small (-0.10) to large (-0.96) effect sizes. Conversely, habitat type had a significant positive effect on plasma BChE activity of shorebirds in North America using Hedges' *d*. Although both cumulative effect sizes were non-significant, the upper confidence interval for the overall effect on AChE was slightly greater than zero. AChE serves an important role in neurotransmission and is generally used to measure exposure to OPs and CBs. Given the number and magnitude of negative species-specific effect sizes on AChE activity, we conclude that shorebirds are exposed to ChE-inhibiting pesticides on the non-breeding grounds.

We found limited evidence for regional variation in ChE activity. Plasma AChE was higher in North America than South America for 1 of 2 species (White-rumped Sandpipers). BChE activity was also higher in North America for Pectoral Sandpipers though the sample size from South America was small. Regional variation might be expected due to three

non-exclusive factors. First, lower AChE activity in South America relative to North America could be due to changes in diet during movements between hemispheres (Goldstein et al. 1999b). Second, the amount of subcutaneous fat may partially explain differences in plasma ChE activity. A significant positive correlation between body fat and serum BChE activity has been reported in human patients (Randell 2005). Last, exposure to other contaminants not measured in this study could be affecting plasma ChE activity. Dieter (1974) found that chronic, low-dose dietary exposure of quail to DDE (a metabolite of the organochlorine DDT), Aroclor 1254 (a polychlorinated biphenyl), and mercuric chloride (a heavy metal) affected plasma TChE activity.

In cases like the Buff-breasted Sandpiper, where plasma ChE activity is lower at an agricultural site where pesticides were recommended for use, reactivation of plasma ChEs *in vitro* can be helpful in determining exposure to ChE inhibitors. However, we were unable to successfully reactivate plasma ChEs by two independent methods. Our results are somewhat inconclusive because some chemicals (i.e., di-methyl OPs) form a stable OP-enzyme complex through dealkylation and resist chemical reactivation. In contrast, some OPs and CBs readily undergo spontaneous reactivation (dephosphorylation or decarbamylation) leading to normally functioning plasma enzymes not long after poisoning, making inhibition difficult to detect (Wilson et al. 1992, Hunt and Hooper 1993). The rate of dealkylation or ageing is usually inversely related to the rate of dephosphorylation (Wilson et al. 1992).

Extraction of chemical residues from footwashes can also provide evidence of exposure to ChE inhibitors. Analysis of footwashings from this study yielded no evidence of dermal exposure to OPs or CBs. We made efforts to sample shorebirds from areas where OPs and CBs were recommended for pest control, but the actual spatial and temporal use patterns of these

pesticides were unknown. The disparity in Buff-breasted Sandpiper plasma ChE activity between reference sites and pesticide use sites in South America deserves further study by residue analyses of prey items collected in the field and assessments of exposure tied to known anti-ChE applications (Hooper et al. 1989, Wilson et al. 1991).

### ***Spatial Variation in Body Mass***

One effect of ChE inhibition is reduced foraging ability and anorexia resulting in decreased body mass (Grue 1982, Grue et al. 1997). We tested for an effect of exposure to OPs and CBs by using body mass as an indicator of fitness, and made comparisons between sites and regions. Although shorebird body mass varied between habitats, our results indicated that shorebirds were able to maintain and even have higher body mass at pesticide use sites. Specifically, we were unable to detect an effect of exposure to ChE inhibitors in Buff-breasted Sandpipers. Regional body mass differences also existed in some species. Individuals sampled in North America had heavier body masses which could be a result of variation in the migratory status of these individuals. Shorebirds may maintain and use large fat stores during migration while they are comparatively lean during stationary periods at non-breeding grounds (Harrington et al. 1991).

### ***Plasma and Brain ChE Activity***

Buff-breasted Sandpiper brain TChE activity (24.3 units/g brain  $\pm$  3.1 SD, n = 6) was similar to values published for other shorebird species. Using similar ChE analysis methods, Mitchell and White (1982) reported 22.8 units/g brain ( $\pm$  5.6 SD, n = 60) for Western Sandpipers, 16.2 units/g brain ( $\pm$  3.4 SD, n = 55) for Long-billed Dowitchers (*Limnodromus scolopaceus*), and 19.4 units/g brain ( $\pm$  4.5 SD, n = 30) for American Avocets (*Recurvirostra americana*) sampled at Laguna Madre, Texas during the non-breeding season. Custer and

Mitchell (1991) reported values between 11.1 and 17.8 units/g brain ( $14.7 \pm 1.8$  SD,  $n = 55$ ) for Willets (*Catoptrophorus semipalmatus*) sampled from the same area.

### **Conclusions**

Our field study is one of the first prospective studies to evaluate exposure to ChE-inhibiting pesticides in migratory species. Overall, most species showed little evidence of exposure. However, our results suggest that Buff-breasted Sandpipers were exposed to ChE-inhibiting pesticides in South America and pesticide use sites could have potentially negative effects on plasma ChE activity in other shorebird species. Buff-breasted Sandpiper samples with depressed ChE levels originated from one field site in Argentina where other species were not captured in large numbers. We suggest continued cooperation and collaboration with investigators and universities in Argentina to continue monitoring of Buff-breasted Sandpiper plasma ChE activity levels at the non-breeding grounds. Further monitoring could also provide more robust sample sizes for species where we provide baseline estimates of ChE activity but lacked complete sampling in this study.

The expansion of agriculture into native habitats will continue to pose a conservation challenge for migratory species. Future research in ecotoxicology of migratory birds requires continued collaboration with international partners to identify local sites of exposure and the initiation of new partnerships to expand monitoring efforts to other sites of interest. The development and use of new pesticide formulations that are less toxic to non-target species and the coordination of pesticide applications to provide maximum benefit to farmers while posing little threat to migratory species are also needed. Additionally, studies using stable isotopes and radio-telemetry can increase our knowledge of habitat use by shorebirds during migration and at

non-breeding sites (Fry et al. 1998, Farmer et al. 2004). This knowledge can help better assess exposure potential and focus future monitoring efforts in high-risk areas.

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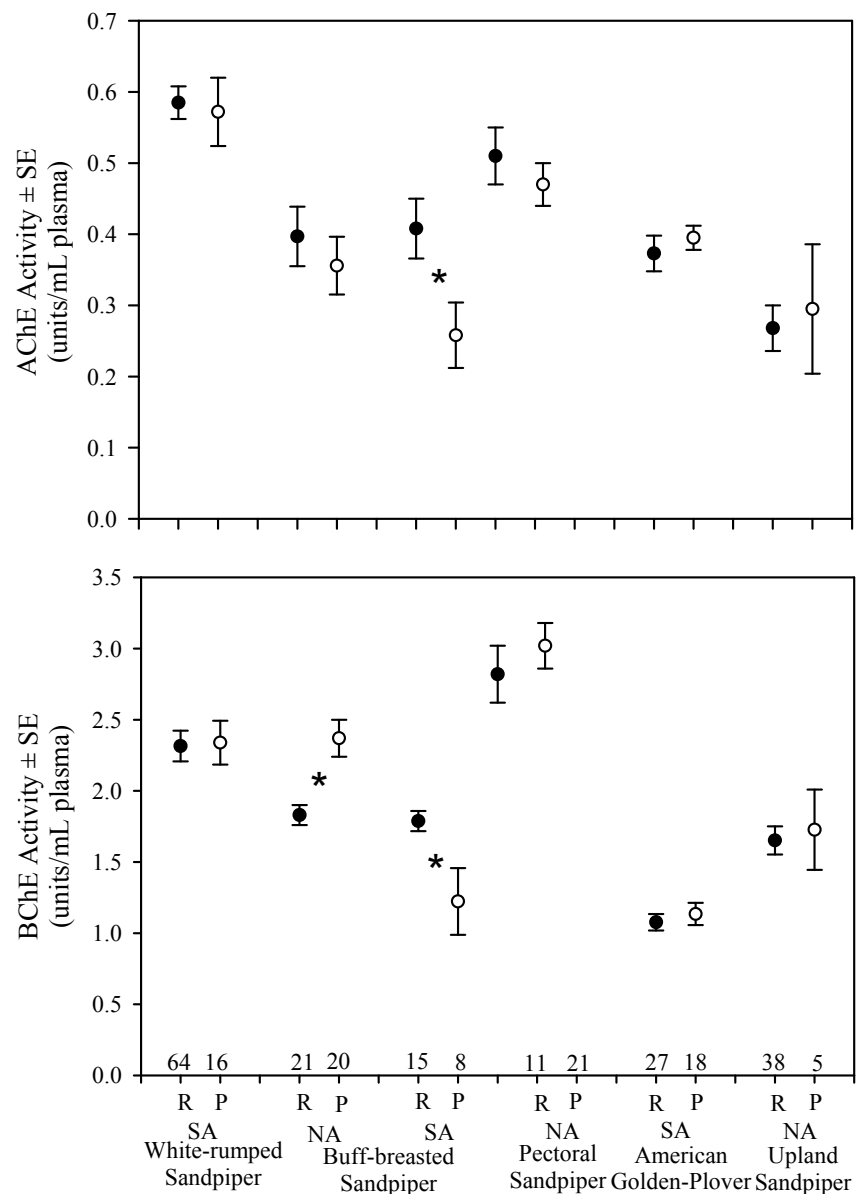


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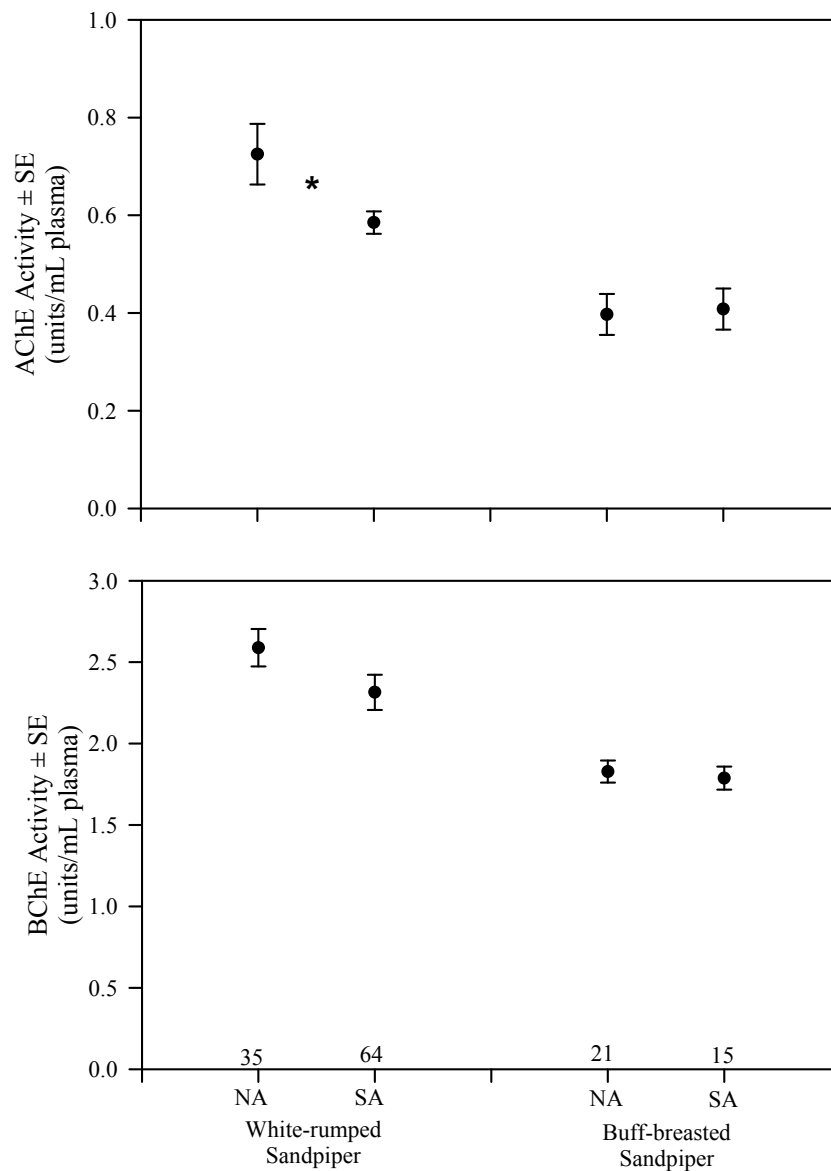
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**Figure 3-1 Pesticide use effects on AChE (above) and BChE (below) of five shorebird species captured in North and South America. Mean (units/mL plasma) and standard error (SE) of ChE activity from individuals sampled from reference sites (R; closed circles) and pesticide use sites (P; open circles). Site of capture, region (NA = North America and SA = South America), and species are located below the lower x-axis. Sample sizes are located above the lower x-axis. An asterisk (\*) denotes a significant difference between means using a two-tailed t-test at an  $\alpha$ -level  $\leq 0.05$ .**



**Figure 3-2 Influence of region on AChE (above) and BChE (below) in two shorebird species. Region of capture (NA = North America, SA = South America) and species are located below the lower x-axis. Sample sizes are located above the lower x-axis. An asterisk (\*) denotes a significant difference between regional means using a two-tailed t-test at an  $\alpha$ -level  $\leq 0.05$ .**



**Table 3-1 Common names and Latin names of study species with sample size (n) of individuals captured in each region.**

Common Name	Latin Name	North America (n)	South America (n)
<i>Nearctic-Neotropical Migrants</i>			
American Golden-Plover	<i>Pluvialis dominica</i>	3	47
Lesser Yellowlegs	<i>Tringa flavipes</i>	3	6
Upland Sandpiper	<i>Bartramia longicauda</i>	67	-
Semipalmated Sandpiper	<i>Calidris pusilla</i>	60	-
Least Sandpiper	<i>Calidris minutilla</i>	65	-
Western Sandpiper	<i>Calidris mauri</i>	76	-
White-rumped Sandpiper	<i>Calidris fuscicollis</i>	36	86
Pectoral Sandpiper	<i>Calidris melanotos</i>	38	42
Stilt Sandpiper	<i>Calidris himantopus</i>	12	6
Buff-breasted Sandpiper	<i>Tryngites subruficollis</i>	67	24
Common Snipe	<i>Gallinago gallinago</i>	3	3
<i>South American Endemics</i>			
Southern Lapwing	<i>Vanellus chilensis</i>	-	6
Collared Plover	<i>Charadrius collaris</i>	-	16
South American Painted-Snipe	<i>Nycticryphes semicollaris</i>	-	5

**Table 3-2 Descriptive statistics of ChE activity (units/mL plasma) by site and effect size of habitat use for 7 species of shorebirds captured in South America. Statistics include sample size (n), mean, standard deviation (SD), minimum (min), and maximum (max). ChE activity was compared between sites using a two-tailed t-test and a measure of effect size, Hedges' *d*. Comparisons were not conducted if  $n \leq 5$ .**

Species	ChE Type	South America									
		Reference					Pesticide Use				
		n	mean	SD	min	max	n	mean	SD	min	max
American Golden-Plover	AChE	27	0.37	0.13	0.18	0.65	18	0.40	0.07	0.26	0.53
	BChE	27	1.08	0.30	0.51	1.71	18	1.14	0.33	0.70	1.77
Lesser Yellowlegs	AChE	6	0.61	0.20	0.32	0.89	-	-	-	-	-
	BChE	6	4.38	1.23	2.99	5.81	-	-	-	-	-
White-rumped Sandpiper	AChE	64	0.59	0.19	0.20	1.05	16	0.57	0.19	0.24	0.94
	BChE	64	2.32	0.87	0.28	4.72	16	2.34	0.61	1.42	3.37
Pectoral Sandpiper	AChE	3	0.52	0.03	0.48	0.54	39	0.39	0.14	0.12	0.86
	BChE	3	1.84	0.32	1.50	2.12	39	2.09	0.53	0.92	3.47
Buff-breasted Sandpiper	AChE	15	0.41	0.16	0.23	0.73	8	0.26	0.13	0.13	0.49
	BChE	15	1.79	0.28	1.07	2.18	8	1.23	0.66	0.40	2.65
Common Snipe	AChE	3	0.31	0.08	0.22	0.37	-	-	-	-	-
	BChE	3	2.26	0.61	1.60	2.82	-	-	-	-	-
South American Painted-Snipe	AChE	3	0.44	0.17	0.28	0.62	2	0.47	0.11	0.39	0.55
	BChE	3	0.89	0.17	0.72	1.05	2	0.58	0.10	0.51	0.65

\* Indicates result is significant using a two-tailed t-test at an  $\alpha$ -level of 0.05

\*\* Indicates result is significant using a two-tailed t-test at an  $\alpha$ -level of 0.01

**Table 3-3 Descriptive statistics of ChE activity (units/mL plasma) by site and effect size of habitat use for 9 species of shorebirds captured in North America. Statistics include sample size (n), mean, standard deviation (SD), minimum (min), and maximum (max). ChE activity was compared between sites using a two-tailed t-test and a measure of effect size, Hedges' *d*. Comparisons were not conducted if  $n \leq 5$ .**

North America															
Species	ChE Type	Reference					Pesticide Use					<i>t</i>	<i>P</i> ≤	<i>d</i>	SD( <i>d</i> )
		N	mean	SD	min	max	n	mean	SD	min	max				
Lesser Yellowlegs	AChE	2	0.57	0.32	0.34	0.79	-	-	-	-	-	-	-	-	-
	BChE	2	3.68	0.85	3.08	4.29	-	-	-	-	-	-	-	-	-
Upland Sandpiper	AChE	38	0.27	0.20	0.01	1.06	5	0.29	0.20	0.06	0.59	0.28	0.78	+0.10	0.48
	BChE	38	1.63	0.61	0.60	3.11	5	1.73	1.41	0.28	1.19	0.35	0.73	+0.13	0.48
Semipalmated Sandpiper	AChE	41	0.50	0.19	0.07	0.89	-	-	-	-	-	-	-	-	-
	BChE	41	8.34	4.54	1.21	20.4	-	-	-	-	-	-	-	-	-
Western Sandpiper	AChE	7	0.48	0.17	0.24	0.76	-	-	-	-	-	-	-	-	-
	BChE	7	5.16	3.59	3.08	13.1	-	-	-	-	-	-	-	-	-
Least Sandpiper	AChE	42	0.47	0.16	0.27	0.99	2	0.43	0.15	0.33	0.54	-	-	-	-
	BChE	42	2.61	0.86	0.81	4.16	2	1.57	0.03	1.55	1.59	-	-	-	-
White-rumped Sandpiper	AChE	35	0.73	0.37	0.30	2.31	-	-	-	-	-	-	-	-	-
	BChE	35	2.56	0.68	1.53	3.99	-	-	-	-	-	-	-	-	-
Pectoral Sandpiper	AChE	11	0.51	0.13	0.32	0.67	21	0.47	0.13	0.26	0.75	-0.70	0.49	-0.30	0.37
	BChE	11	2.82	0.67	1.21	3.95	21	3.02	0.74	1.77	4.43	0.73	0.47	+0.27	0.37
Buff-breasted Sandpiper	AChE	21	0.40	0.19	0.16	0.89	20	0.35	0.18	0.11	0.99	-0.80	0.43	-0.26	0.32
	BChE	21	1.83	0.31	1.43	2.56	20	2.31	0.56	1.00	3.40	3.47	0.01**	+1.05	0.33
Common Snipe	AChE	3	0.35	0.06	0.32	0.42	-	-	-	-	-	-	-	-	-
	BChE	3	2.06	0.85	1.39	3.02	-	-	-	-	-	-	-	-	-

\*\* Indicates result is significant using a two-tailed t-test at an  $\alpha$ -level of 0.01



**Table 3-4 Mean body mass (g) of 13 species of shorebirds by site and region. Body mass means that share the same letter superscript are not significantly different within a species using a two-tailed t-test at an  $\alpha$ -level  $\leq 0.05$ . Superscripts A and B are comparisons within regions (within a row) and superscripts Y and Z are comparisons between sites (within a column). Comparisons were not conducted if  $n \leq 5$ .**

Common Name	Mass (g)											
	North America						South America					
	Site*	n	Mean	SD	min	max	Site*	n	Mean	SD	min	max
<i>Nearctic-Neotropical Migrants</i>												
American Golden-Plover	R	3	141.0	10.4	134.0	153.0	R	28	131.0 <sup>Y</sup>	7.5	103.0	155.0
	-	-	-	-	-	-	P	18	135.0 <sup>Y</sup>	9.2	119.0	149.0
Lesser Yellowlegs	R	3	100.0	19.5	80.0	119.0	R	6	91.5	24.4	72.0	139.0
Upland Sandpiper	R	62	149.0 <sup>Y</sup>	20.6	118.0	212.0	-	-	-	-	-	-
	P	5	131.0 <sup>Z</sup>	9.8	124.0	148.0	-	-	-	-	-	-
Semipalmated Sandpiper	R	60	29.4	5.4	20.5	42.0	-	-	-	-	-	-
Least Sandpiper	R	62	23.1	3.6	18.0	36.0	-	-	-	-	-	-
	P	3	19.3	0.6	19.0	20.0	-	-	-	-	-	-
Western Sandpiper	R	76	26.9	2.9	22.0	39.0	-	-	-	-	-	-
White-rumped Sandpiper	R	36	37.7 <sup>A</sup>	4.5	31.0	50.0	R	70	35.2 <sup>B,Y</sup>	3.2	25.5	45.0
	-	-	-	-	-	-	P	16	37.3 <sup>Z</sup>	3.0	33.0	45.0
Pectoral Sandpiper	R	13	58.7 <sup>Y</sup>	13.7	44.5	90.0	R	3	49.6	2.1	48.0	52.0
	P	25	77.8 <sup>Z</sup>	13.0	53.0	92.0	P	39	63.2	11.9	35.5	86.0
Stilt Sandpiper	R	12	54.0 <sup>A</sup>	9.8	44.0	72.0	R	6	53.5 <sup>A</sup>	2.1	51.0	56.0
Buff-breasted Sandpiper	R	22	63.4 <sup>A,Y</sup>	7.5	46.0	79.0	R	15	55.1 <sup>B,Y</sup>	5.8	42.0	62.0
	P	45	70.6 <sup>Z</sup>	12.7	42.0	101.0	P	9	61.1 <sup>Z</sup>	5.4	50.5	65.5
Common Snipe	R	3	118.0	5.2	115.0	121.0	R	3	121.0	5.9	117.0	128.0
<i>South American Endemics</i>												
Collared Plover	-	-	-	-	-	-	R	5	32.8 <sup>Y</sup>	3.6	29.0	37.0
	-	-	-	-	-	-	P	11	32.6 <sup>Y</sup>	3.6	26.0	37.5
South American Painted-Snipe	-	-	-	-	-	-	R	3	64.6	3.8	62.0	69.0
	-	-	-	-	-	-	P	2	63.0	5.7	59.0	67.0

\*R = Reference site, P = Pesticide use site

## CHAPTER 4 - Conclusions

Migratory shorebird populations face on-going global population declines. Effective conservation of shorebird populations requires an understanding of factors affecting shorebirds throughout their annual cycle. Shorebirds cover vast geographic expanses during migration and at the non-breeding grounds. Likewise, population limiting factors can be encountered at any stage. My thesis addressed one potential cause for migratory shorebird declines, exposure to environmental contaminants at the non-breeding grounds. Specifically, I estimated exposure to organophosphorus (OP) and carbamate (CB) pesticides during migration and at non-breeding sites and tested for an effect of exposure by comparing body mass between habitats. I addressed variability of the specific biomarker, cholinesterase (ChE), used to determine exposure to these chemicals. I provide the first comprehensive data available on exposure of migratory shorebirds to ChE-inhibitors and provide important information regarding conservation of these species.

Variability in plasma ChEs is important when estimating exposure to OPs and CBs across different spatial and temporal scales. I found that shorebird plasma ChEs decreased as body size increased. Thus, comparisons of enzyme activity must be conducted separately for each species. Intrinsic factors such as sex and body condition and extrinsic factors such as time of capture and date of capture can also affect ChE activity. In North America, plasma butyrylcholinesterase (BChE) increased with date of capture in two species, Upland Sandpipers and Least Sandpipers and plasma acetylcholinesterase (AChE) increased with capture date in one species, White-rumped Sandpipers. Sex differences were found in only one species, the Upland Sandpiper; females had higher BChE than males. Time of capture and body condition did not affect ChE activity in any species tested in North America. Regionally, White-rumped

Sandpiper plasma AChE was higher in North America. My results indicate the importance of evaluating environmental and biological influences on plasma ChEs in field toxicology studies.

I assessed exposure of migratory shorebirds to ChE inhibitors in five species by comparing ChE activity of samples collected at reference sites to samples collected from agricultural areas during the same temporal and spatial framework. I found evidence for exposure to ChE-inhibitors in South America in one species, the Buff-breasted Sandpiper. In contrast, plasma ChEs were higher at use sites in North America for the same species. There were no differences in plasma ChE activity between habitats in any other species tested. Using a meta-analysis, I determined that agricultural habitats had a negative effect on AChE activity in 4 of 6 comparisons, an indication that sub-lethal exposure may be occurring in other migratory shorebird species. However, the cumulative effect sizes were non-significant. My results suggest that some species of migratory shorebirds sampled in this study were exposed to ChE-inhibiting pesticides at the non-breeding grounds.

The conversion of natural habitats to agricultural land uses will continue to pose a conservation threat to migratory shorebirds. While shorebirds can exploit available resources in agricultural habitats, exposure to agrochemicals is a threat to the conservation of at least one species of migratory shorebird. I recommend continued monitoring of Buff-breasted Sandpipers and other shorebird species in more diverse agricultural habitats and continued cooperation with international partners. Future research should include interviews with farmers to increase knowledge of local pesticide use. Initiation of education campaigns to educate farmers about the toxicity of OPs and CBs and to devise alternative pest management regimes is needed. Monitoring should also be expanded to other agricultural regions of the Americas that are

important to non-breeding shorebirds. These measures will ensure thorough attention to this conservation issue.

Although I made preliminary estimates of exposure to OPs and CBs in non-breeding shorebirds, I did not evaluate behavior of exposed individuals. Thus, I could not make inferences about the potential behavioral effects of sub-lethal exposure. Exposure-induced behavioral changes can have important consequences on individual condition and survival. Additionally, my study addressed exposure of migratory shorebirds to one type of environmental contaminant, ChE-inhibiting pesticides. Other studies have focused on heavy metals and DDT metabolites at the non-breeding grounds (e.g., see Chapter 3) but effects of these contaminants have received little attention at the breeding grounds. Contaminants may have independent or synergistic negative effects on individuals that could decrease fecundity. Research on the breeding grounds should incorporate exposure to contaminants as a variable in reproductive and behavioral studies.

Overall, my data provides insight into one anthropogenic source of shorebird population declines. Habitat loss and degradation and illegal hunting may also contribute to population declines. Shorebirds exploit a variety of habitats during migration and at the non-breeding grounds, however the amount of time spent in and the relative importance of each habitat is unknown. Using tools such as stable isotopes and radio telemetry we can infer foraging vs. roosting habitat and estimate exposure risk to ChE inhibitors and other anthropogenic sources of declines. This will help determine the significance of these risk factors to population declines of migratory shorebirds.

## **Appendix A - Laboratory controls and inter-lab variation**

Horse serum was used as a laboratory standard to compare within and between lab variation at Kansas State University (KSU) and the Texas Institute for Environmental and Human Health (TIEHH). There was no difference in mean  $\pm$  SD standard serum activity between labs for either AChE (KSU:  $19.3 \pm 5.9$  SD,  $n = 53$ ; TIEHH:  $18.6 \pm 7.0$  SD,  $n = 46$ ;  $t_{97} = 0.47$ ,  $P = 0.64$ ) or BChE (KSU:  $221.7 \pm 22.2$  SD,  $n = 53$ ; TIEHH:  $222.6 \pm 10.4$  SD,  $n = 46$ ;  $t_{76} = -0.25$ ,  $P = 0.80$ , [unequal variance]) when temperature, substrate, and inhibitor concentrations were identical  $1.0 \times 10^{-4}$  M). This comparison of standards validates the use of ChE activity from different labs in combined statistical analyses. Overall, within lab variation was higher for BChE activity measured at KSU (10.0% CV) than TIEHH (4.7% CV).

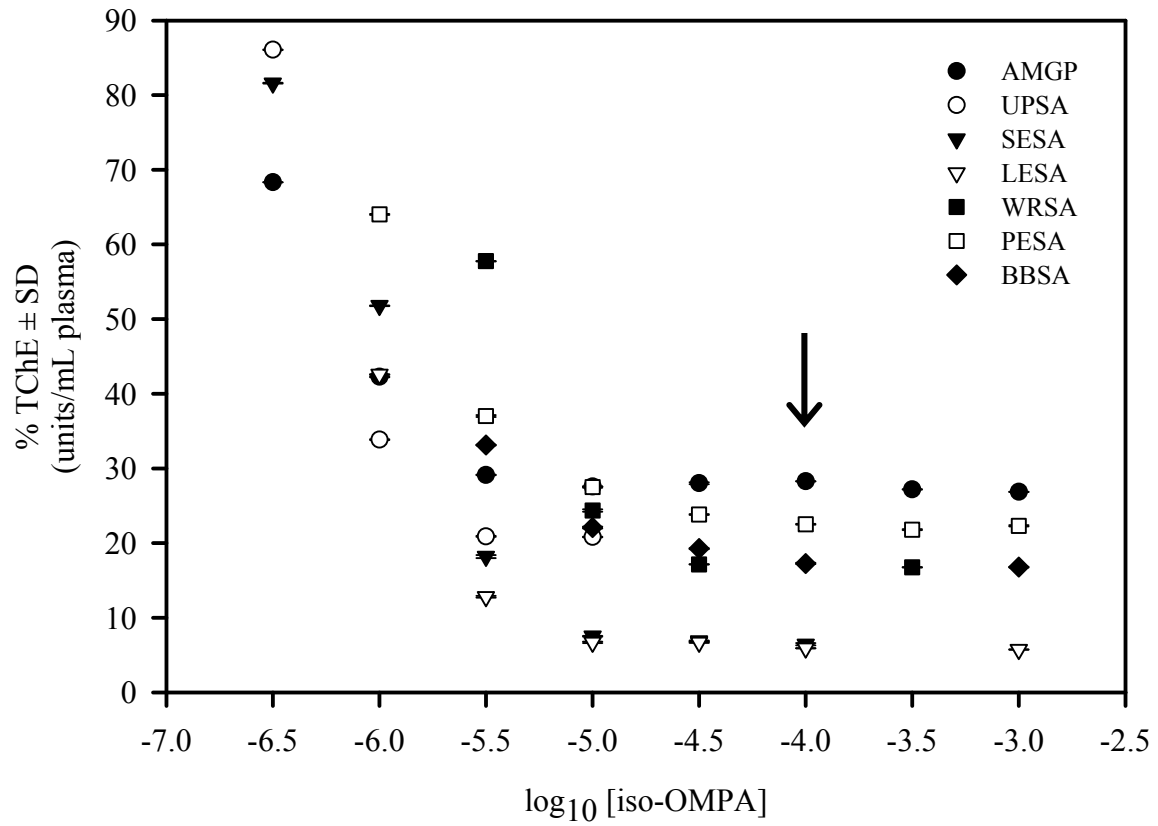
## **Appendix B - Characterization of shorebird plasma cholinesterases for optimal inhibitor and substrate concentrations**

As a first step, plasma ChEs were characterized for optimal inhibitor (iso-OMPA) and substrate (AThCh) concentrations. Iso-OMPA is a BChE-specific inhibitor and is used to separate AChE and BChE activity. BChE activity is calculated as the difference between total cholinesterase (TChE) measured without iso-OMPA and AChE activity in the presence of iso-OMPA. Ideal concentrations of iso-OMPA are sufficient to inhibit all BChE without inhibiting AChE activity. The optimal concentration of iso-OMPA is found within the plateau region of a graph depicting %TChE Activity vs.  $\log_{10}[\text{iso-OMPA}]$ . In this study the optimal concentration of iso-OMPA was similar for all species,  $1.0 \times 10^{-4}$  M (Fig. B-1, arrow).

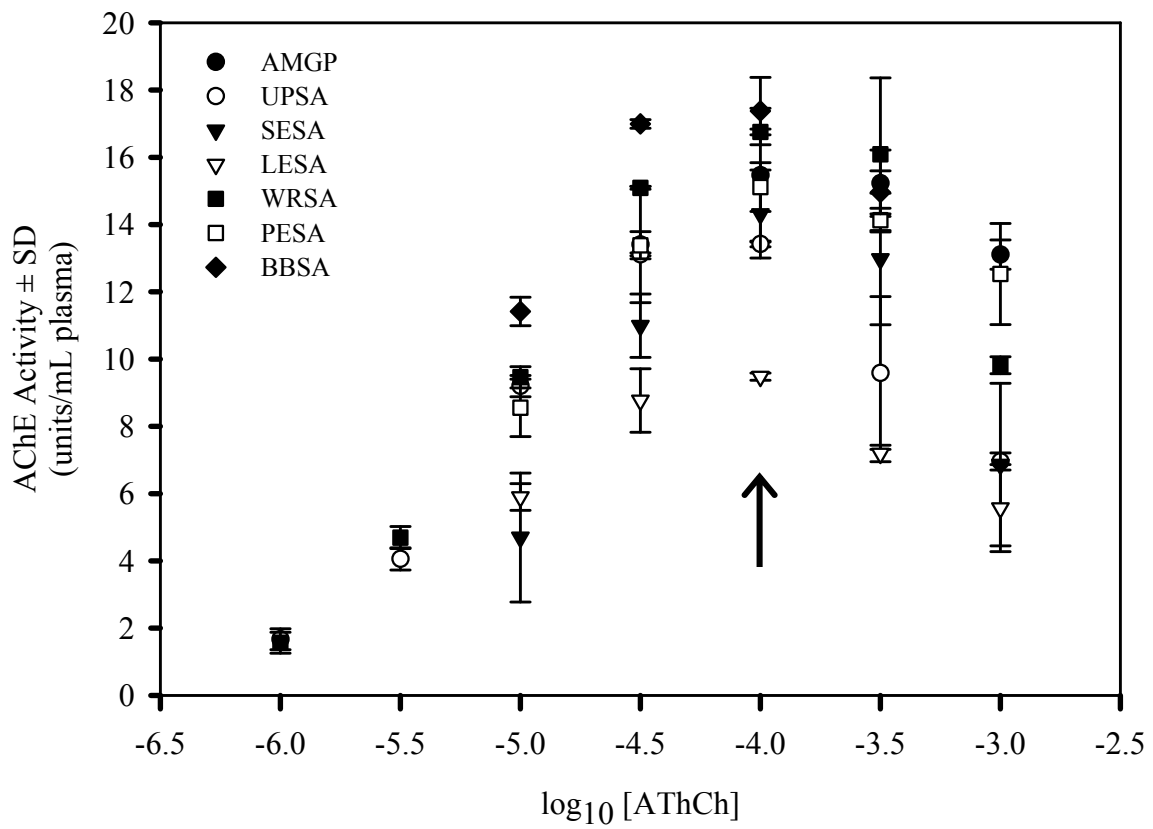
Similarly, Substrate concentration must be chosen to maximize enzyme activity without inhibiting sample enzyme. Optimal substrate concentrations are found at the peak of the bell-shaped curve of AChE activity vs.  $\log_{10}[\text{AThCh}]$ . Optimal substrate concentration was similar for all species,  $1.0 \times 10^{-4}$  M (Fig. B-2, arrow).

Species abbreviations in Figures B-1 and B-2 are as follows: AMGP = American Golden-Plover, UPSA = Upland Sandpiper, SESA = Semipalmated Sandpiper, LESA = Least Sandpiper, WRSA = White-rumped Sandpiper, PESA = Pectoral Sandpiper, BBSA = Buff-breasted Sandpiper.

**Figure B-1 Iso-OMPA titration curves of 7 species of migratory shorebirds.**



**Figure B-2 Substrate (AThCh) affinity curve of 7 species of migratory shorebirds.**





## Appendix C - Plasma cholinesterase activity of additional species

Nine species of shorebirds lacked sufficient data for inclusion in Chapters 2 and 3. Reference values of ChE activity of 7

Neartic-Neotropical migratory shorebird species were not included in Chapter 2 because  $n \leq 5$ . Two species of South American endemics were sampled only at pesticide use sites or had only one sample collected from reference sites and were not included in Chapter 3.

**Table C-1 Plasma ChE activity (units/mL plasma) of 9 shorebird species sampled at migratory stopover and non-breeding sites with insufficient data for statistical analyses.**

Common Name	Scientific Name	Site*	n	AChE			BChE			
				mean	SD	min	max	mean	SD	min
<i>Nearctic-Neotropical Migrants</i>										
Snowy Plover	<i>Charadrius alexandrinus</i>	R	1	0.391	-	-	-	1.895	-	-
Spotted Sandpiper	<i>Actitis macularis</i>	R	4	0.710	0.116	0.547	0.806	1.494	0.532	2.264
Solitary Sandpiper	<i>Tringa solitaria</i>	R	1	0.811	-	-	-	1.183	-	-
Baird's Sandpiper	<i>Calidris bairdii</i>	R	1	0.439	-	-	-	3.184	-	-
Dunlin	<i>Calidris alpina</i>	R	2	0.695	0.431	0.390	1.000	2.631	0.019	2.618
Long-billed Dowitcher	<i>Limnodromus scolopaceus</i>	R	1	1.235	-	-	-	1.258	-	-
Wilson's Phalarope	<i>Phalaropus tricolor</i>	R	2	0.725	0.173	0.603	0.848	6.200	1.776	4.944
<i>South American Endemics</i>										
Southern Lapwing	<i>Vanellus chilensis</i>	P	6	0.229	0.066	0.161	0.317	1.158	0.561	0.647
Collared Plover	<i>Charadrius collaris</i>	R	1	0.326	-	-	-	1.183	-	-
		P	5	0.278	0.083	0.155	0.383	1.158	0.513	0.647
										2.096

\*R = Reference site, P = Pesticide use site

## **Appendix D - Analyses of variation in plasma cholinesterases of migratory shorebirds using additional samples**

Sex differences and diurnal variation in plasma ChE activity of shorebirds sampled during north-bound migration in 2006 were reported in Chapter 2. The following analyses were conducted according to statistical methods described in Chapter 2 but contain additional data from samples collected during south-bound migration in North America and at non-breeding sites in South America in 2006.

Plasma BChE was 22.4% higher in female Upland Sandpipers than males (Table D-1). No other differences between sexes were detected in AChE or BChE. These results are consistent with previous analyses (see Chapter 2).

There was significant variation in plasma BChE activity in 1 of 7 species tested (Table D-2). Semipalmated Sandpiper BChE activity (units/mL plasma) was higher in individuals captured between 06:00 – 11:59h ( $11.6 \pm 4.5$  SD,  $n = 14$ ) than individuals captured between 12:00 – 17:59h ( $4.4 \pm 1.9$  SD,  $n = 4$ ) or 18:00 – 23:59h ( $6.9 \pm 3.7$  SD,  $n = 22$ ). No other species showed variation in plasma ChE activity with time of capture.

**Table D-1 Differences in plasma ChE activity by sex in 2 species of shorebirds sampled at migratory stopover sites in North America. Results were considered significant at a  $\alpha$ -level of 0.05 using a two-tailed t-test.**

Species	ChE Type	Region	Female			Male			<i>t</i>	df	<i>P</i> ≤
			n	Mean	SD	n	Mean	SD			
Upland Sandpiper <sup>†</sup>	AChE	NA	16	0.26	0.19	22	0.27	0.21	-0.1	36	0.925
	BChE		16	1.87	0.64	22	1.45	0.52	2.18	36	0.037*
Semipalmated Sandpiper <sup>‡</sup>	AChE	NA	29	0.49	0.22	9	0.53	0.21	-0.6	36	0.580
	BChE		29	8.61	4.85	9	7.02	4.05	0.89	36	0.381

<sup>†</sup>Sexed using molecular markers; <sup>‡</sup>Sexed using Prater et al. 1977

\*Indicates a significant result at an  $\alpha$ -level ≤ 0.05

**Table D-2 Diurnal variation in plasma ChEs of 7 species of shorebirds sampled at reference sites during migration in North America and at non-breeding sites in South America.**

**Results were considered significant at an  $\alpha$ -level of 0.05 if  $P < 0.006$  after sequential Bonferroni correction for the number of tests.**

Species	Region	ChE Type	Time of Capture		
			df	<i>F</i>	<i>P</i> ≤
American-Golden Plover	SA	log(AChE)	1,25	1.2	0.284
		log(BChE)	1,25	1.6	0.212
Upland Sandpiper	NA	log(AChE)	1,36	4.8	0.035
		log(BChE)	1,36	0.1	0.716
Buff-breasted Sandpiper	NA	log(AChE)	1,19	0.0	0.858
		log(BChE)	1,19	0.8	0.373
	SA	log(AChE)	1,12	2.5	0.139
		log(BChE)	1,12	4.6	0.053
White-rumped Sandpiper	NA	log(AChE)	1,32	0.1	0.750
		log(BChE)	1,32	0.2	0.653
	SA	log(AChE)	1,62	2.8	0.102
		log(BChE)	1,62	0.5	0.485
Pectoral Sandpiper	NA	log(AChE)	1,9	0.6	0.466
		log(BChE)	1,9	0.0	0.864
Least Sandpiper	NA	log(AChE)	1,37	0.1	0.800
		log(BChE)	1,37	0.2	0.697
Semipalmated Sandpiper	NA	log(AChE)	1,38	0.2	0.701
		log(BChE)	1,38	8.5	0.006*

\*Indicates a significant result at an  $\alpha$ -level  $\leq 0.05$